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Field protocols for the genomic era

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Abstract. For many decades karyotype was the only source of overall genomic information obtained from species of mammal. However, approaches have been developed in recent years to obtain molecular and ultimately genomic information directly from the extracted DNA of an organism. Molecular data have accumulated hugely for mammalian taxa. The growing volume of studies should motivate field researchers to collect suitable samples for molecular analysis from various species across all their ranges. This is the reason why we here include a molecular sampling procedure within a field work protocol, which also includes more traditional (including cytogenetic) techniques. In this way we hope to foster the development of molecular and genomic studies in non-standard wild mammals.

Key words: mammalian genome, molecular phylogeography, field studies, molecular sampling procedure, karyotype, *Sorex araneus*.

Introduction

For many decades karyotype was the only source of overall genomic information that could be obtained from species of mammal. Up to now, karyotyping has been based on the original technique known as "A colchicine, hypotonic citrate, squash sequence for mammalian chromosomes" - a title of the most frequently cited paper of C.E. Ford and J.L. Hamerton (1956). Recent researchers continue to practice the same technique with just two differences - sodium citrate is replaced by potassium chloride as a hypotonic solution, and air-drying is used instead of squashing. In general, this procedure is used widely and has been adapted both for field and laboratory studies.

More recently, there has been a development of molecular methods which will ultimately lead to whole-genome information. In considering wild mammals, much of this work has involved specific DNA sequences obtained from extracted DNA of an organism, and following the phylogeographic approach to understand the distribution of such sequences and phylogenetic lineages in a geographic context (Avise, 2000; Beheregaray, 2008). The growing volume of phylogeographic studies and more recent approaches involving a much larger proportion of the genome, means that field researchers should collect material in a way that is useful for such molecular work. In this paper we provide these molecular protocols together with other (including cytogenetic) methods that a field mammalogist may follow in their standard studies of multiple species within their geographic area.

Our experience in collecting field samples for molecular studies has been gained mostly from the investigation of chromosome races

Fig. 1. ISACC emblem with the image of a common shrew and its characteristic sex chromosomes (XY_1Y_2 system). Designed by J. Hausser (Mém. Soc. Vaud. Sc. Natur. 1991. 19, see citation for Searle et al., 1991).

of the common shrew (*Sorex araneus* Linnaeus, 1758). This small insectivorous mammal has been the subject of particularly detailed study since 1987 when the International *Sorex araneus* Cytogenetics Committee – ISACC – was formed (Fig. 1, Searle et al., 2007). The standardisation of procedures and datasets achieved through ISACC has included designation of the standard *Sorex araneus* karyotype (Searle et al., 1991), rules to define chromosome races (Hausser et al., 1994) and the generation of comprehensive lists of chromosome races (Zima et al., 1996; Wójcik et al., 2003). New needs require some additional protocols and standardisation. This is the reason why we considered it appropriate to include a molecular sampling procedure into a set of standardised field protocols, which we trust will be of value as a reference for future studies and a springboard for future extensive genomic research on wild mammals.

Genaral Remarks

The common shrew, or, in Latin, *Sorex araneus* Linnaeus, 1758 – a small terrestrial insectivore – got its scientific name from the famous Sweden naturalist Carl von Linné who lived and worked in the 18th century in the vicinity of the ancient university town of Uppsala. Though his own field researches were addressed primarily to wild and cultivated

Fig. 2. Portrait of Carl von Linné painted in 1732 (in Lapland dress and with the flower named after him, *Linnea borealis*).

plants (see Fig. 2), many species of mammal from around the world were included as Linnean species in the great nature catalogues indefatigably compiled by him.

In the next century in England, Charles Darwin generated the extraordinary "Origin of Species" whose 150 years anniversary we are celebrating in 2009 together with the bicentenary of Darwin's birth. It took nearly a full century after the "Origin" before the structure of DNA was discovered (1953) and when modern mammalian cytogenetics originated (1956 – see above). It took another half-century before genome analysis has become possible for wild mammals, although molecular approaches such as "phylogeography" predate that (Avise, 2000).

In its original definition, "phylogeography is a field of study connected with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species. As the word implies, phylogeography deals with historical, phylogenetic components of the spatial distribution of gene lineages" (Avise, 2000, p. 3). The empirical basis for this field grew from mitochondrial (mt) DNA research on field material. So, it is important to obtain samples containing well conserved DNA. Fortunately, the protocol for sample collection is very simple, although it does need care. It relates both to samples for mtDNA and nuclear DNA analysis. Because of the difficulty in obtaining high quality DNA from museum specimens, fresh samples are enormously important.

Field Procedures

Species to collect

Each project is primarily focussed on a certain species such as the common shrew in our case. However, during fieldwork, inevitably other small mammal species may be collected which also can be kept and documented. We will not comment here on details relating to animal capture. It is an aspect of fieldwork separate from our molecular and cytogenetic focus!

Samples for DNA may be obtained from a killed animal at once, or following approximately half an hour, just after the initial part of the chromosome procedure, if the animal is being karyotyped. Different body parts such as feet, tail or ear, as well as internal organs like liver, spleen or heart may be sampled (Fig. 3). It is important to keep the samples of different organs taken from the same animal in different tubes though they can be removed with the same instruments. The liver (excluding bile) is such a large organ that it can be stored in several tubes (useful for specimen exchange).

If there are no special sampling needs relating to a particular species, 1 to 3 individuals should be collected per trapping locality. Other individuals can be released. The specimens should be given an appropriate code used on the specimen label and in field and laboratory notebooks (Table 1). Details should be recorded of the species identification, sex, collection site and collector's name. The geographical coordinates of collection sites should be taken using GPS, if possible.

External measurements of animal

Record the following after killing the animal (Table 1):

1) male/female
2) adult/immature
3) for females, record whether mated (sometimes evident because of a nape scar or white patch), lactating or pregnant
4) weight to nearest 0.1 g
5) head + body length to nearest 0.1 mm
6) tail length to nearest 0.1 mm
7) length of right hind foot to nearest 0.1 mm.

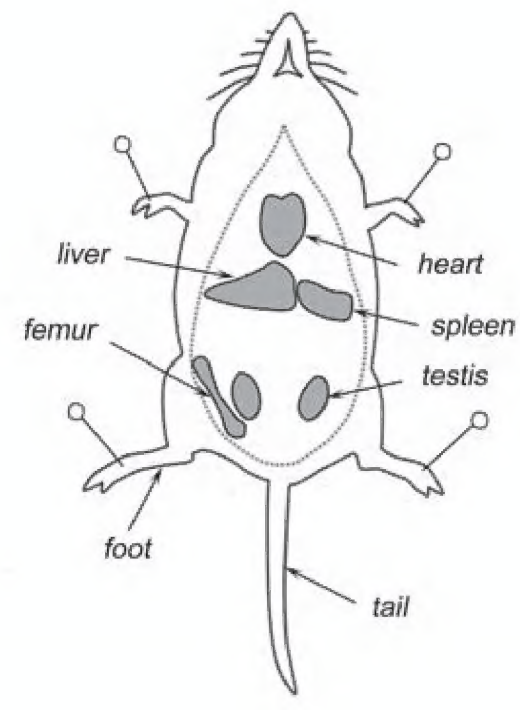

Fig. 3. Tissue sources used for DNA and cytogenetic studies.

Table 1. An example of the data collected on a small mammal used for cytogenetic and molecular studies (viz. the shrew specimen whose karyotype is presented in Fig. 4). * BL – head and body length, mm / TL – tail length, mm / HL – hindfoot length, mm / BM - body mass, g.

Specimen code	**1**	06-06N
Date	**2**	27.07.06
Species	**3**	*S. araneus*
Sex	**4**	♂
Age	**5**	Imm
Locality	**6**	Seredysh island, Middle Volga River, Toljatti Distr., E E Russia (53°28'N/ 49°45'E)
Body size* BL/TL/HL/BM	**7**	67,1/39,2/12,3/-
Karyotype	**8**	+
DNA samples	**9**	Liver, foot
Description	**10**	Karyotype
References	**11**	Bystrakova N., Nadjafova R., Pavlova S. New data on distribution of chromosome races of the common shrew (*Sorex araneus* L.) – Mologa and Neroosa // Theriofauna of Russia and adjacent territories. VIII meeting of the Theriological Society. Moscow 2007. P. 64.
Other information	**12**	Skull (NBY pers. coll.)

Whole-body specimens

It is valuable that individuals collected are preserved permanently for museum or laboratory collections. There are different ways to conserve wet or dry specimens depending on specialized personal requirements. Once the specimen is adequately sampled for DNA (see below), it is extremely convenient to preserve specimens in formalin, because this is an excellent preservative and non-inflammable. However, it must be emphasized that this procedure is only appropriate for those body parts that are not needed for molecular analysis. Thus, the specimens may be prepared by removing the guts and internal organs, putting a tag on the hind leg and storing in a container of formalin in the ratio 1 shrew: 10 formalin. These formalin-preserved specimens are easily transported by removing excess formalin and placing those individuals to be sent in a well-sealed plastic bag.

Samples for DNA analysis

For each individual we collect at least two samples for DNA preparations.

1) Part of one of the feet including the toes. The foot should be cut with clean instruments (ideally use a disposable razor blade – which removes any danger of cross-contamination), and stored in a 1.5 ml screw-top plastic tube. Fill the tube with 95-100% ethanol (pure ethanol rather than methylated spirits); there should be at least 10 times as much ethanol as tissue. Make sure that the tube is adequately labelled (e.g. with pencil; the label should not wash off with ethanol). Store at 4°C if possible (otherwise room temperature).

2) The tail tip or ear may be collected in the same way.

3) Soft tissue from the heart, spleen or part of the liver. The animal should be dissected with clean instruments. The heart should be chopped into small pieces with clean instruments (ideally use a disposable razor blade – which removes any danger of cross-contamination). The preservation of tissue follows (1) thereafter.

Please note that it really is critical to have clean instruments when preparing samples

for DNA preparations. It is important not to have cross-contamination from one specimen to another. This is why disposable razor blades are so good. If you are careful there is no need to touch the tissue with any other instruments. Scissors and forceps will carry DNA from previous specimens if they have only been washed with water or ethanol. It is possible to clean DNA from instruments but it is a laborious process: Wipe the instruments, soak them in a 10% bleach solution, rinse very thoroughly with water (if bleach is present on the instruments when you next use them – that will destroy the DNA of the next specimen!), squirt ethanol on all sides of the instruments and flame them.

Chromosome preparations

Working with cell suspensions has formed the primary strategy for chromosome preparations since its first description by C.E. Ford and J.L. Hamerton in 1956. The original basic procedure involves an *in vivo* colchicine pretreatment of the specimen, which may also be applied to cultivated cells or post mortem extracted bone marrow cells *in vitro*. It includes the following stages:

1) Inject 0.5 ml of 0.025 % (w/v) colchicine solution intraperitoneally. Leave the animal for 1 h.

2) Kill the animal by cervical dislocation. Remove femurs. Cut off epiphyses. Wash out the marrow into a small vial with warm 1.12% (w/v) sodium citrate solution, using a hypodermic syringe with a fine needle. [Nowadays: 0.56% potassium chloride is preferred as a hypotonic solution].

3) Gently aspirate marrow in and out of the syringe until it is broken up into a fine suspension. Place the vial containing the suspension into a water bath at 37°C. Leave for 10 min.

4) Filter by centrifuging through Nylon bolting cloth in a bacterial filtration tube. [Nowadays: precipitate cells by centrifuging 5 min at 1000 rpm in a benchtop centrifuge].

5) Fix in chilled acetic-alcohol (1:3), 2 h.

Figure 4 shows an example of the final product of chromosome preparation after air-drying. It is a karyotype of the common shrew, illustrating both polymorphism and polytypy, and the chromosomes have been stained and arranged in a standard format for the species (Searle et al., 1991).

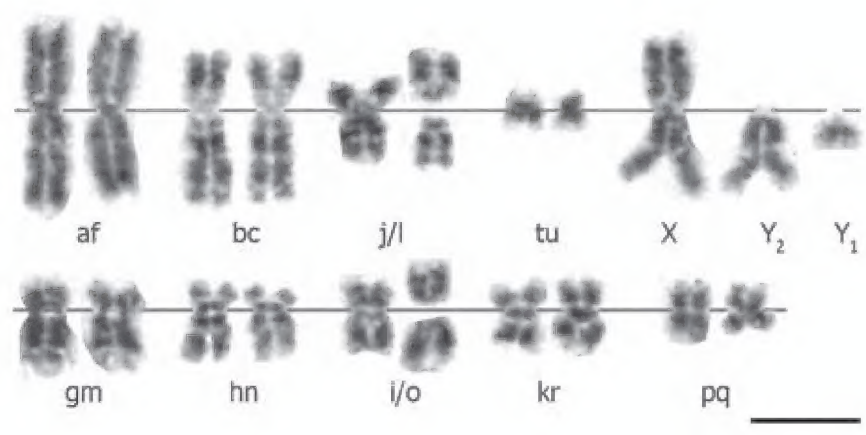

Fig. 4. A karyotype generated from a bone-marrow preparation of a male common shrew made in the field. G-band staining allows each chromosome to be identified (Searle et al., 1991) and the karyotype to be assigned to a particular chromosome race (Wójcik et al., 2003); in this case the Penza race (Penza Region, East European Russia). This karyotype not only illustrates polytypy in the common shrew, but also polymorphism; note that the individual is heterozygous for chromosomes *io* and *jl* and that there is a particular nomenclature developed to describe that (Searle et al., 1991). Because the individual is a male it shows the unusual multiple sex chromosomes, XY_1Y_2. Bar=10 μm.

Meiosis

The analysis of meiosis is an advanced form of chromosomal study that is valuable, for instance, in hybrid zones between chromosome races, where heterozygous individuals (see Fig. 4) are found. It is situations where there are configurations more complicated than bivalents that meiosis becomes interesting. Figure 4 illustrates another interesting sit-

uation to examine meiosis: multiple sex chromosomes. Most straightforward is to make meiotic preparations in males, making use of the paired testes (Fig. 4). To make the meiotic chromosome preparations for late prophase I (diakinesis) – metaphase II, the testis tubules should be removed from the tunica and kept in a small Petri dish in hypotonic 1% citrate at room temperature. Then the tubules should be transferred into a vial with acetic-ethanol (1:3) fixative using forceps and kept there for 10 min. The fixative solution should be replaced three times. Place the vial into the refrigerator for a period up to 2 weeks. To make slides, take a very small mass of tubules, homogenize them with scissors as finely as possible and place a few drops onto a slide (Grafodatsky, Radjabli, 1988). To obtain preparations for the analysis of earlier meiotic stages (in particular, to examine chromosome pairing by visualising synaptonemal complexes at pachytene), intact testes should be placed in 10 volumes of cold isotonic solution and forwarded to a laboratory specialised for such studies.

Cell Culture

Potentially it is also possible to collect tissue samples for *in vitro* fibroblast culture in the field. In this case it is very important to follow certain rules strictly, first of all with respect the maintenance of sterility and the transport of samples without exposing them to extremes of temperature when sending them to a laboratory that will set up the culture. The tissues samples should only be taken from freshly killed individuals.

Acknowledgments

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